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Extensive Chromatin Remodeling and Establishment of Transcription Factor 'Hotspots' during Early Adipogenesis

Rasmus Siersbaek, Ronni Nielsen, Sam John, Myong-Hee Sung, Songjoon Baek, Anne Loft, Gordon L. Hager and Susanne Mandrup

Corresponding author: Susanne Mandrup, University of Southern Denmark

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 04 November 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has been now been evaluated by three referees and I enclose their reports below, as you will see they currently provide mixed recommendations regarding publication.

Overall the majority of referees find that the analysis of chromatin remodeling events and transcription factor hotspots during adipogenesis to be interesting and important. After discussing the reports with the referees a subset of the concerns need to be experimentally addressed. The issues concerning the role of C/EBPbeta and delta on regulating PPARgamma activity should be tested by depletion of C/EBP, as should the role of the co-dependence of the transcription factors involved in the hotspots (raised by all referees), and the biological consequence of not forming these hotspots. Two of the referees also request a link to gene expression changes during differentiation. However, there are two issues that do not need to be specifically addressed. These concerns the comments raised by referee #2 on the description of previous work on chromatin changes during adipogenesis, I appreciate that many of the listed papers mentioned by this referee describe changes in chromatin modifications rather than actual remodeling events, so while these comments should be taken into consideration they do negatively influence the novelty of the current study. In addition referee #3 also asks that different cell lines/ differentiation protocols be used to generalize the findings, I am aware that many studies utilize this method to induce adipogenesis and therefore, find that analysis of different protocols is not, in this instant, required for publication here. After careful consideration the main concerns raised here are central to the conclusions proposed in the manuscript and addressing them would very much strengthen the study. Given the interest in the study should you be able to address these issues, we would be happy to consider a revised manuscript.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

This is a very interesting paper, which uses DHS-seq to explore for the first time the genome-wide changes in chromatin structure that occur during adipogenesis. The studies are well-performed and analyzed, and the finding of genome-wide chromatin remodeling during the initial hours of adipogenesis is novel. The DHS data led to the discovery of sites (termed hotspots), which were found to be bound by multiple transcription factors. C/EBPbeta is clearly shown by bioinformatic analysis and by knockdown experiments to have an important role in organizing these hotspots. While these are strong points in favor of this work, the paper would be improved by relating the binding of C/EBPbeta back to the strongest point of the paper, namely the extensive changes in chromatin structure. In addition it would be important to compare the relative importance of C/EBPbeta with other factors implicated in the hotspots, such as GR and STAT5. These points are detailed below:

1. Page 7, the statement "The relatively low numbers of DHS sites detected at day 0 and day 6 are due to the fact that the cells at these stages are non-proliferating" is stated as fact, when really this is conjecture and would be better suited to the Discussion (where a similar statement is also made on page 17).

2. Page 12: The suggestion that C/EBPβ and -δ may directly regulate PPARγ activity at a subset of PPARγ target sites by regulating chromatin accessibility early in adipogenesis should be tested by knockdown of the C/EBPs, to directly test this hypothesis. This should be readily accomplished since C/EBPbeta knockdown is used on page 15 and Fig. 6 to evaluate its necessity for recruitment of other transcription factors.

3. Page 15: Knockdown of C/EBPbeta reduced binding of STAT5 and GR, and from this the authors conclude that "C/EBPβ is specifically required for efficient binding of these transcription factors to shared sites early in adipocyte differentiation." This conclusion is appropriate based on the data, but it is not clear whether C/EBPbeta is uniquely driving the binding of the other factors, or whether there is mutual dependence, for example of the other factors on the binding of C/EBPbeta. Indeed, Steger et al (2010) did show that GR knockdown reduces C/EBPbeta binding to selected sites at early times points during adipogenesis. Do the authors see the same co-dependence of C/EBPbeta on GR in this system? What about STAT5- does its knockdown alter C/EBP and GR binding? If so, how does that effect the conclusion that C/EBP is a pioneer- would the other factors be also considered "pioneers"? Or are they somehow "co-pioneers"? Depending on the results of such experiments, this should be discussed.

Referee #2 (Remarks to the Author):

This paper reports the use of DNase-Seq to characterize chromatin remodeling events during 3T3-L1 adipogenesis. The key findings are:

1. Tens of thousands of DHS were identified at various time points of differentiation, with more occurring early than late.

2. PPARg appears to bind late to many sites that may already be open.

3. C/EBPb and C/EBPd bind early and often in the developing adipocyte, and may act as pioneer factors for later binding by downstream TFs, such as PPARg.

4. DHS that open early in adipogenesis are associated with GR and STAT sites. ChIP of GR and STAT5a confirms their binding to these regions and demonstrates functional cooperativity between GR, STAT5, and C/EBPs.

5. Knockdown of C/EBPb prevented binding of many GR, STAT, and RXR sites.

This is a nice paper that adds to our current knowledge of the epigenetic regulation of adipogenesis (and differentiation in general), especially as regards early events. I have a few scientific concerns, and then an additional significant concern about the tone of the paper.

1. * Pg 7 - "The relatively low numbers of DHS sites detected at day 0 and day 6 are due to the fact that the cells at these stages are non-proliferating".

* Pg 17 - "... the accumulation of fat in adipocytes interferes with DNase I digestion, thereby reducing the number of identified DHS sites in mature adipocytes".

The discussion of the total number of DHS sites at each time point throughout the paper is problematic for several reasons. (1) The claim that non-proliferating cells have less DHS sites is stated as fact, yet it is not backed up by data or appropriate citations. (2) The authors give both biological and technical explanations for why the number might be low in adipocytes. They clearly favor the biological explanation for their model, yet no data are provided that can be used to judge their relative importance. (3) Only a single DHS library appears to have been sequenced for each time point. It is therefore impossible to judge how robust the DHS site counts really are. There is no a priori reason to expect that two independent DHS-Seq libraries will provide the same sensitivity and specificity. For the comparison of DHS site counts between time points to be valid, the authors must show that it is robust to experimental variation and the specific thresholds chosen for each library.

2. * Pg 25 - "False discovery rate calculations"
The randomization-based thresholding method employed by the authors is a reasonable ad hoc method, but it has nothing to do with the method described in the paper they cite (Benjamini and Hochberg, 1995). Moreover, because the threshold is calculated from a single randomization using what is probably an unrealistically simple null model, it does not provide a conservative FDR estimate. It is therefore inappropriate to refer to the threshold as the "FDR 0% level".

3. I like the experiment to reduce C/EBPb and test the effect on GR and STAT binding. What would happen if you knocked down GR or STAT instead? In other words, the primacy of C/EBPb is inferred from an experiment in which the alternative hypothesis (GR and STAT regulate C/EBPb binding) is not tested.

4. No correlations are made with gene expression. Was mRNA expression not measured as part of this experiment? It would be useful to know whether genes showing transient induction of DHS spots show transient gene expression, for example.

5. One of the most interesting things about DNAse hypersensitivity as a tool for looking at

epigenetic change is the ability to infer specific TF binding sites by deeper sequencing, in effect 'footprinting' the precise cis-element being occupied. This has been done in other systems and could offer unique insights not available through other means. Has this been considered?

And finally, a comment on the tone of the paper:

* Pg 4 - "Nothing is known about the chromatin remodeling events that are required for the development of adipocytes".

* Pg 5 - "This study provides the first genome-wide map of chromatin transitions through a differentiation process and reveals a novel direct crosstalk between early and late events during adipocyte differentiation"

* Pg 16 - "In this study, we present the first genome-wide maps of the changes in chromatin structure that accompany a differentiation process."

These assertions are simply untrue. I am quite certain that the authors are aware of the literature in this field, but among others Steger et al. Genes and Development (2010), Mikkelsen et. al. Cell (2010), and Lefterova et al. Mol and Cell Biol (2010) have characterized chromatin remodeling events during adipogenesis. In particular, Lefterova et al describe transient events involving chromatin remodeling, GR and CEBPb binding that are very similar to those described in this manuscript. Mikkelsen et al. has presented an extensive collection of genome-wide chromatin state maps from differentiating 3T3-L1 and human adipocytes. This is not even the first look at chromatin remodeling in adipocytes using DNAse hypersensitivity-Eguchi et. al. (2008) used that approach as well (albeit not on a genome-wide scale). As to the first point above, there are also numerous papers looking at chromatin remodeling events in adipogenesis that focus on specific loci, including several papers by the groups of Imbalzano and Sakai. There are even reviews on this subject (Organogenesis. 2010;6:24). I would encourage the authors to take a more generous view of the field in future versions of this and other manuscripts.

Referee #3 (Remarks to the Author):

In this manuscript the authors address the genomic changes that occur during adipogenic differentiation of the 3T3-L1 cell line. Using high-throughput sequencing the presence of DNAseI hypersensitive sites and binding sites for relevant transcription factors (C/EBPbeta, C/EBPdelta, RXR, STAT5) at various timepoints during the differentiation time course. Bioinformatics is used to identify genes co-regulated by two or more TFs and to identify clusters of temporal regulation of occupancy. Finally, based on these results, knockdown of C/EBPbeta is shown to decrease RXR, GR and STAT5 binding to co-occupied genes early in the differentiation process.

1. The interpretation of the data (early, late genes etc) is heavily biased by the induction protocol (2 days with cAMP agonist/glucocorticoid, then removal of these). There are massive changes occurring (cell cycle, cell shape) that are not necessarily linked to adipogenesis. Looking at clusters etc. without taking this into account is simplistic. All the clusters are biased by the high number of early DHSs, and it seems unlikely that late-induced genes do not exist at all.

2. Similarly, the interpretation of the sequence of events needs to be free of bias due to the pharmacological method. C/EBPbeta/delta are directly induced by the cAMP/Dex cocktail at early timepoints, and PPARgamma is not significantly up-regulated at that stage, so the sequence is not surprising. However, would use of PPARgamma agonists to induce differentiation produce a similar endpoint with different intermediate kinetics? If general conclusions about the fundamantal mechanisms of adipogenesis are to be reached it is necessary to go beyond a specific pharmacological cell culture model.

3. The co-localization of multiple TFs at DHSs is interesting, but also for these exprements the issues raised in points 1+2 need to be considered.

4. It is a general problem that there is no association of the ChIP/DHS data to gene expression is

included. Which are the genes expressed in terminally differentiated cells relative to the identified TF binding sites? What are their cellular functions?

5. The data on recruitment of GR/RXR/STAT5 in C/EBPbeta knockdown cells is potentially interesting. However, there is no demonstartion that this is physiologically relevant. The C/EBPbeta-deficient population should be better characterized, including any changes in expression of other TFs, adipogenic potential (preferably including rescue experiments) to show that the loss of C/EBPbeta is the direct cause of the observed change.

Overall, the manuscript contains a number of interesting observations, but most of these are still in an incomplete state, and the general relevance beyond the specific cell line/pharmacolocigal model used remains to be demonstrated.

1st Revision - authors' response 01 February 2011

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Response: We agree with the reviewer that this is conjecture and we have therefore removed the statement.

2. Page 12: The suggestion that C/EBPβ and -δ may directly regulate PPARγ activity at a subset of PPARγ target sites by regulating chromatin accessibility early in adipogenesis should be tested by knockdown of the C/EBPs, to directly test this hypothesis. This should be readily accomplished since C/EBPbeta knockdown is used on page 15 and Fig. 6 to evaluate its necessity for recruitment of other transcription factors.

Response: We agree that it would indeed be interesting to experimentally investigate the importance of early C/EBP binding for late PPARgamma binding. However, as C/EBPs are required for induction of PPARgamma expression during 3T3-L1 adipocyte differentiation, knockdown of C/EBPbeta and -delta would impair induction of PPARgamma expression and adipogenesis. Thus, unfortunately this particular question cannot be addressed. However, what we can address is the role of C/EBPbeta in the binding of other transcription factors to common binding sites and in early chromatin remodeling. We show that C/EBPbeta is important for the binding of transcription factors to shared sites including 'hotspots' (revised Fig. 7) and for efficient chromatin remodeling (Suppl. Fig. 8).

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Response: This is an excellent point. We have knocked down GR and Stat5a using the same approach as for the C/EBPbeta knock down to investigate the importance of these TFs for establishment of TF 'hotspots' (new Fig. 8). We find that both GR and Stat5a are important for efficient binding of other TFs (including C/EBPbeta) to selected 'hotspots', indicating highly cooperative binding of TFs to these sites. Thus, in this regard C/EBPbeta does not play a dominant role, except that it cooperates with many different transcription factors at more sites than the other factors investigated. However, the intriguing observation for C/EBPbeta is that it binds a large number of sites including 'hotspots' already at day 0. It is this ability of C/EBPbeta to bind closed chromatin and facilitate the binding of other adipogenic transcription factors induced by the hormonal cocktail, which indicates that C/EBPbeta is a pioneering factor.

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Response: We agree with the reviewer that the statement regarding the number of fewer DHS sites in non-proliferating cells was too speculative and have now removed this statement. However, we are confident of our results regarding the fewer DHS sites at day 0 and day 6, since we have performed DHS-seq for day 0 and day 6 from an independent differentiation. We find a high concordance between the two replicates (very similar numbers of binding sites and more than 70% overlap between the two data sets) demonstrating that the data presented here are robust to experimental variation.

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The randomization-based thresholding method employed by the authors is a reasonable ad hoc method, but it has nothing to do with the method described in the paper they cite (Benjamini and Hochberg, 1995). Moreover, because the threshold is calculated from a single randomization using what is probably an unrealistically simple null model, it does not provide a conservative FDR estimate. It is therefore inappropriate to refer to the threshold as the "FDR 0% level".

Response: We agree with the reviewer that it is not entirely correct to refer to the threshold as the 'FDR 0% level', and we have changed the Methods section accordingly. We now state that we have used FDR=1/N, where N is the number of observed hotspots. This gives FDR<1.1E-4 in this study.

3. I like the experiment to reduce C/EBPb and test the effect on GR and STAT binding. What would happen if you knocked down GR or STAT instead? In other words, the primacy of C/EBPb is inferred from an experiment in which the alternative hypothesis (GR and STAT regulate C/EBPb binding) is not tested.

Response: This is an excellent point. We have knocked down GR and Stat5a using the same approach as for the C/EBPbeta knock down to investigate the importance of these TFs for establishment of TF 'hotspots'. We show that both GR and Stat5a are important for binding of other TFs to selected 'hotspots', indicating cooperative binding of TFs to these sites. These data are now presented in a new Fig. 8. See also response to reviewer 1 point 3.

4. No correlations are made with gene expression. Was mRNA expression not measured as part of this experiment? It would be useful to know whether genes showing transient induction of DHS spots show transient gene expression, for example.

Response: We agree that it is interesting to correlate the development of DHS sites with changes in gene expression. We have previously used RNA Pol II ChIP-seq to monitor transcriptional changes during 3T3-L1 adipogenesis. We have now used this data set to group genes according to their mode of transcriptional regulation (induced, transiently induced, repressed and constitutive) and have subsequently asked which types of DHS sites are enriched in the vicinity of the different gene groups(addition to Fig. 2)(Suppl. Table 1 for GO analysis of groups). We find that the development of DHS sites is highly correlated with transcriptional changes of nearby genes during adipogenesis. In addition, we have added new RNA Pol II data from day 0 and 4 hours and correlated early transcriptional activation with binding of transcription factors at 4 hours (new Fig. 6)(Suppl. Table 2 for GO analysis of groups). 'Hotspots' are highly enriched in the vicinity of early induced genes, and interestingly, of the factors tested GR appears to play a particular important role.

5. One of the most interesting things about DNAse hypersensitivity as a tool for looking at epigenetic change is the ability to infer specific TF binding sites by deeper sequencing, in effect 'footprinting' the precise cis-element being occupied. This has been done in other systems and could offer unique insights not available through other means. Has this been considered?

Response: This is an interesting point. However, to robustly identify TF footprints using DHS-seq, the samples would need to be sequenced very deep (approx. 100-150 M tags for mouse and human genomes). We consider such in-depth analyses of transcription factor footprints beyond the scope of this study.

And finally, a comment on the tone of the paper:

** Pg 4 - "Nothing is known about the chromatin remodeling events that are required for the development of adipocytes".*

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Response: We agree with the reviewer that this is not the first paper describing changes to the chromatin template during adipogenesis. We definitely did not mean to claim that. Several other groups have profiled epigenetic marks at a genome-wide scale at different stages of adipocyte differentiation (Steger et al. Genes and Development 2010, Lefterova et al MCB 2010, Wakabayashi et al MCB 2009), and after the submission of our manuscript Mikkelsen et al. Cell 2010 published a very comprehensive study describing the development of multiple histone marks during adipocyte differention. We have used the term 'chromatin structure' and 'chromatin remodeling' to describe the overall structure of chromatin (i.e. the degree to which the chromatin structure is open/closed) and not the histone modifications associated with the chromatin structure. We have changed the text to clarify this and to reference the important work on the epigenetic profiles. Similarly, we have changed the text to acknowledge the studies on chromatin remodeling of specific loci assessed by nuclease accessibility. We agree with the reviewer that it is not correct to say that 'nothing is known about remodeling' during adipocyte differentiation, and we changed this statement to 'little is known'.

To investigate the correlation between the development of histone marks and the structural changes as assessed by DHS, we have correlated the DHS sites identified in this study with histone marks identified by Mikkelsen et al 2010 (H3K4me1, H3K4me3, H3K27ac, and H3K27me3) (Suppl. Fig. 1). As is seen from these analyses most distal and promoter DHS sites at day 2 are associated with chromatin marks known to be enriched at enhancers and promoters, respectively, whereas there is little overlap with the repressive mark H3K27me3. Thus, these analyses agree with our current understanding of which chromatin marks are enriched in open chromatin regions. Notably, however, there are a large number 'active chromatin marks' outside DHS regions. Furthermore, there is only limited correlation between the development of chromatin marks and DHS sites (Suppl. Fig. 1D-E).

In addition, we have determined the overlap between regions transiently enriched in the active chromatin mark H3K9Ac at day 1 of 3T3-L1 differentiation reported by Steger et al 2010 (Suppl. Fig. 2) and DHS sites in the four different clusters. We show that the majority of the DHS sites located in these regions having transient H3K9ac are found in the transient cluster 2 and 3, which have maximal DHS sensitivity at 4 hours and day 1, respectively.

Taken together, these analyses show clear correlations between activating histone marks and DHS, as has previously been reported. However, it is also evident that the DHS and active histone marks represent different types of chromatin signatures with dissimilar temporal profiles.

Referee #3 (Remarks to the Author):

In this manuscript the authors address the genomic changes that occur during adipogenic differentiation of the 3T3-L1 cell line. Using high-throughput sequencing the presence of DNAseI hypersensitive sites and binding sites for relevant transcription factors (C/EBPbeta, C/EBPdelta, RXR, STAT5) at various time points during the differentiation time course. Bioinformatics is used to identify genes co-regulated by two or more TFs and to identify clusters of temporal regulation of occupancy. Finally, based on these results, knockdown of C/EBPbeta is shown to decrease RXR, GR and STAT5 binding to co-occupied genes early in the differentiation process.

1. The interpretation of the data (early, late genes etc) is heavily biased by the induction protocol (2 days with cAMP agonist/glucocorticoid, then removal of these). There are massive changes occurring (cell cycle, cell shape) that are not necessarily linked to adipogenesis. Looking at clusters etc. without taking this into account is simplistic. All the clusters are biased by the high number of early DHSs, and it seems unlikely that late-induced genes do not exist at all.

Response: We agree that the massive early induction of chromatin remodeling is biased by the hormone cocktail. However, this protocol to differentiate 3T3-L1 cells is standard and necessary for efficient differentiation, and a similar protocol is also used for other pre-adipocyte cell lines and primary cells. The great advantage of the 3T3-L1 cell culture model, and one of the reasons for its extensive use, is the ability to undergo a rather synchronous differentiation. We have stressed in the first paragraph of the introduction that the 3T3-L1 cells represent a model system for adipocyte differentiation. Furthermore, we would like to point out that we do actually identify a cluster of regions that develop an open chromatin structure late in adipogenesis and these sites are heavily enriched in the vicinity of late adipocyte genes linked to adipocyte biology.

2. Similarly, the interpretation of the sequence of events needs to be free of bias due to the pharmacological method. C/EBPbeta/delta are directly induced by the cAMP/Dex cocktail at early timepoints, and PPARgamma is not significantly up-regulated at that stage, so the sequence is not surprising. However, would use of PPARgamma agonists to induce differentiation produce a similar endpoint with different intermediate kinetics? If general conclusions about the fundamantal mechanisms of adipogenesis are to be reached it is necessary to go beyond a specific pharmacological cell culture model.

Response: The hormone cocktail is standard and required for efficient and synchronous adipocyte differentiation (see above). While it would be interesting to investigate whether adipocyte differentiation achieved in a different cell culture model or with a modified protocol display the same final structure; we feel this would be beyond the scope of this manuscript.

3. The co-localization of multiple TFs at DHSs is interesting, but also for these exprements the issues raised in points 1+2 need to be considered.

Response: Same as above.

4. It is a general problem that there is no association of the ChIP/DHS data to gene expression is included. Which are the genes expressed in terminally differentiated cells relative to the identified TF binding sites? What are their cellular functions?

Response: We agree that it is interesting to correlate DHS/ChIP and gene expression. See response to reviewer 2 (point 4) above. However, we would also like to stress that our analysis goes beyond genes and concerns all changes in chromatin structure, irrespective of whether these are close to genes or not, that occur during adipocyte differentiation of 3T3-L1 cells.

5. The data on recruitment of GR/RXR/STAT5 in C/EBPbeta knockdown cells is potentially interesting. However, there is no demonstartion that this is physiologically relevant. The C/EBPbeta-deficient population should be better characterized, including any changes in expression of other TFs, adipogenic potential (preferably including rescue experiments) to show that the loss of C/EBPbeta is the direct cause of the observed change.

Response: We agree with the reviewer that it is important to ascertain that the observed changes in the binding of transcription factors to 'hotspots' are caused by loss C/EBPbeta binding to these sites rather than by indirect effects. We have therefore in all ChIP analyses included control sites to which the factor of interest, but not C/EBPbeta, binds. As can be seen in Fig. 7, we never observed an effect of C/EBPbeta knockdown on control sites. As to the physiological role of C/EBPbeta knockdown, several previous studies have demonstrated that C/EBPbeta (and to a lesser extent C/EBPdelta) is important for early steps of adipocyte differentiation both in vivo and in vitro (Linhart *et al.*, 2001;Tanaka *et al.*, 1997;Wang *et al.*, 1995).

Overall, the manuscript contains a number of interesting observations, but most of these are still in an incomplete state, and the general relevance beyond the specific cell line/pharmacolocigal model used remains to be demonstrated.

2nd Editorial Decision 17 February 2011

I received the last report this morning, both referees who have seen the revised version of the manuscript recommend publication. Referee #2 has one very minor text change to recommend (see below). I suggest that you make the change in the word file and email it to us then we can replace it, this then avoids the entire submission process. I am happy to accept the manuscript for publication in The EMBO Journal.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

The authors have satisfactorily addressed all of my concerns.

Referee #2

No major concerns.

One minor point:
When discussing C/EBP "binding to PPARg target sites" (p.12), it might be worth explaining that C/EBP does not actually bind to the PPRE motif, but to sites nearby that fall under the PPARg peak. This may not be apparent to non-aficionados of ChIP-Seq or DNase-Seq.

